ISSN 1479-2621

Plant Genetic Resources **3**(2); 149–169
DOI: 10.1079/PGR200573

Patterns of essential oil relationships in *Pimpinella* (Umbelliferae) based on phylogenetic relationships using nuclear and chloroplast sequences

Nurhayat Tabanca[1], Andrew W. Douglas[2], Erdal Bedir[1,3], Franck E. Dayan[4], Nese Kirimer[5], K. Husnu Can Baser[5], Zeki Aytac[6], Ikhlas A. Khan[1,7] and Brian E. Scheffler[4]*

[1]*National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, University of Mississippi, MS 38677, USA,* [2]*Harding Lab, New York Botanical Garden, 200th St. and Kazimiroff Blvd., Bronx, NY 10458, USA,* [3]*Department of Bioengineering, Faculty of Engineering, Ege University, Bornova, Izmir 35100, Turkey,* [4]*USDA-ARS-NPURU, University, MS 38677, USA,* [5]*Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, 26470 Eskisehir, Turkey,* [6]*Department of Biology, Faculty of Science and Letters, Gazi University, 06500 Ankara, Turkey and* [7]*Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677, USA*

Received 27 September 2004; Accepted 21 April 2005

Abstract

Hydro-distilled essential oils from fruits, aerial parts (without fruits) and roots of *Pimpinella* species native to Turkey and their phylogenetic relationships to one another were examined. Phytochemical investigation of the essential oils of 19 species resulted in isolation of 16 phenylpropanoids, four sesquiterpenes and two azulene-type norsesquiterpenes. The structures of the isolated compounds were determined primarily from 1D- and 2D-NMR experiments as well as liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry. Phylogenetic relationships among 26 species were evaluated using ITS 1, ITS 4 nuclear rDNA and psbA-trnH cpDNA sequences. In this study, significance and occurrence of phenylpropanoids, azulenes and geijerenes are discussed from a phylogenetic, chemical and biosynthetic perspective. The distribution of different classes of compounds and their putative associations with one another as per current knowledge of their biosynthetic pathways indicates that this information, in conjunction with the phylogeny, could provide valuable information regarding the presence and perhaps evolution of the different classes of compounds. Analysis of the phenylpropanoid components indicates that (*E*)-anethole is an obligatory intermediate of this pathway. The various *Pimpinella* species differ primarily in their ability to acylate anethole, suggesting that while the pathway leading to anethole is common to this genus, species differ in their enzymatic machinery leading to acylate. The relationship between azulenes and geijerenes is not as intuitive, but all *Pimpinella* species analysed in this study have the biochemical machinery required to synthesize these chemical classes.

* Corresponding author: USDA-ARS MSA Genomics Laboratory, Stoneville, MS 38776, USA. E-mail: bscheffler@ars.usda.gov

Keywords: *Apiaceae*; essential oils; internal transcribed spacer; molecular phylogeny; norsesquiterpenes; phenylpropanoids; *Pimpinella*; PsbA-trnH spacer

Introduction

Many species in *Pimpinella* L. (*Apiaceae*, Umbelliferae) are agronomically important, particularly those with distinctive aromas and flavours. The most widely known and cultivated *Pimpinella* species is *P. anisum* (common name aniseed; Grieve, 1970a, b; Crellin and Philpott, 1990). Both the extract and essential oil (aniseed being the most commonly known) of *P. anisum* have carminative, expectorant, sedative, antidepressant, antiseptic, insecticidal, antiviral, antispasmodic, nematocidal, mutagenic, analgesic, antifungal, antibacterial, diuretic, pectoral, tonic or stimulant properties (Ross *et al.*, 1980; Duke, 1985; Shashikanth and Hosono, 1986; Twaij *et al.*, 1987; Alman *et al.*, 1988; Lokar and Poldini, 1988; Bisset, 1994; Kiuchi, 1995; Baser, 1997; Miller and Murray, 1998).

In Central Turkey, fresh leaves of endemic *P. anisetum*, locally known as 'Ezeltere', are used on salads and its seeds are used in pickling. It has also been used as a cigar to promote expectoration. Other *Pimpinella* species growing in eastern and south-eastern Turkey are used as animal feed to increase milk production. In the German Pharmacopoeia, *P. major* and *P. saxifraga* are known as 'Pimpinellae Radix' and are used as an expectorant and increase bronchial secretions (Bohn *et al.*, 1989). The root of *P. major* is commercially available in Austria, and aqueous extracts of the root have been reported to have antibacterial activity (Branther and Grein, 1994).

Earlier investigations of *Pimpinella* species resulted in the isolation of terpenoids, lipids, alkaloids, coumarins, flavonoids and phenylpropanoids (Kleiman and Spencer, 1982; Haranath *et al.*, 1985; Kubeczka *et al.*, 1985; Xue *et al.*, 1992; Kubeczka, 1997; Kollmannsberger *et al.*, 2000). Some classes of interesting essential oils of *Pimpinella* species are given in Fig. 1. Both the extract and essential oils of *Pimpinella* species are known to have a high content of phenylpropanoid derivatives. The 2-hydroxy-5-methoxy-1-(*E*)-propenylbenzene skeleton of these compounds, known as pseudoisoeugenol, is unique to *Pimpinella* (Kubeczka, 1997). Antigermination, insecticidal, acaricidal, weak antitumour, antimalarial, antifungal, antimicrobial and oestrogen activities of phenylpropanoids from *Pimpinella* have been reported (Marcus and Lichtenstein, 1979; Kleiman *et al.*, 1988; Reichling and Merkel, 1991; Tabanca *et al.*, 2003, 2004, 2005). The distributions of the compounds, although intriguing, have not been investigated within a phylogenetic framework.

Pimpinella is a member of the *Apiaceae* and comprises approximately 150 species distributed in the northern hemisphere (Constance, 1991). *Pimpinella* is represented in Turkey by 23 species (five endemic), two subspecies and two varieties and 27 taxa altogether (Matthew, 1972; Pimenov and Leonov, 2004). Drude (1897–98) divided *Apiaceae* into three subfamilies, Hydrocotyloideae, Saniculoideae and Apioideae, the latter of which is the largest and includes *Pimpinella* as well as putatively related genera *Anethum*, *Angelica*, *Ferula*, *Foeniculum*, *Heracleum* and *Seseli*. Recent molecular phylogenetic analyses of *Apiaceae* have contributed much to our understanding of evolutionary relationships in *Apiaceae* (Downie *et al.*, 1998, 2000a, b, c, 2001). Downie *et al.* (1998) reported that *Pimpinella* is a member of a weakly supported 'Apium clade' that includes *Anethum*, *Ridolfia*, *Foeniculum*, *Apium*, *Conium*, *Smyrniopsis*, *Prangos* and *Capnophyllum*. Subsequent work by Downie *et al.* (2001) recognizes *Pimpinella* as a distinct clade within the 'Apioid superclade'.

Different species of *Pimpinella* can be annuals, biennials and perennials and are generally characterized by the presence of fibrous collars at the top of the rootstock. There is variation present among the species in fruit shape (ovoid-oblong to subglobose), which helps define *Pimpinella*, petal colour, as well as leaf, bract and bracteole characteristics and these characters have been useful in the classification and identification of species in Turkey (Matthew, 1972). There has not been a detailed phylogenetic analysis of species of *Pimpinella* but, in this study, it is presumed to be a monophyletic group until evidence indicates otherwise.

The aims of this study are: to carry out phytochemical studies on 19 species of *Pimpinella* growing in Turkey; establish a phylogeny of 26 *Pimpinella* species using gene sequence data; use the phylogeny as a framework to ascertain whether there is a connection between the chemical and genetic profiles or not; and finally determine the biosynthetic significance of phenylpropanoids and norsesquiterpenes.

Materials and methods

Plant material

Twenty-six species of *Pimpinella* and an outgroup (*Foeniculum vulgare*) were used for this study. Plant

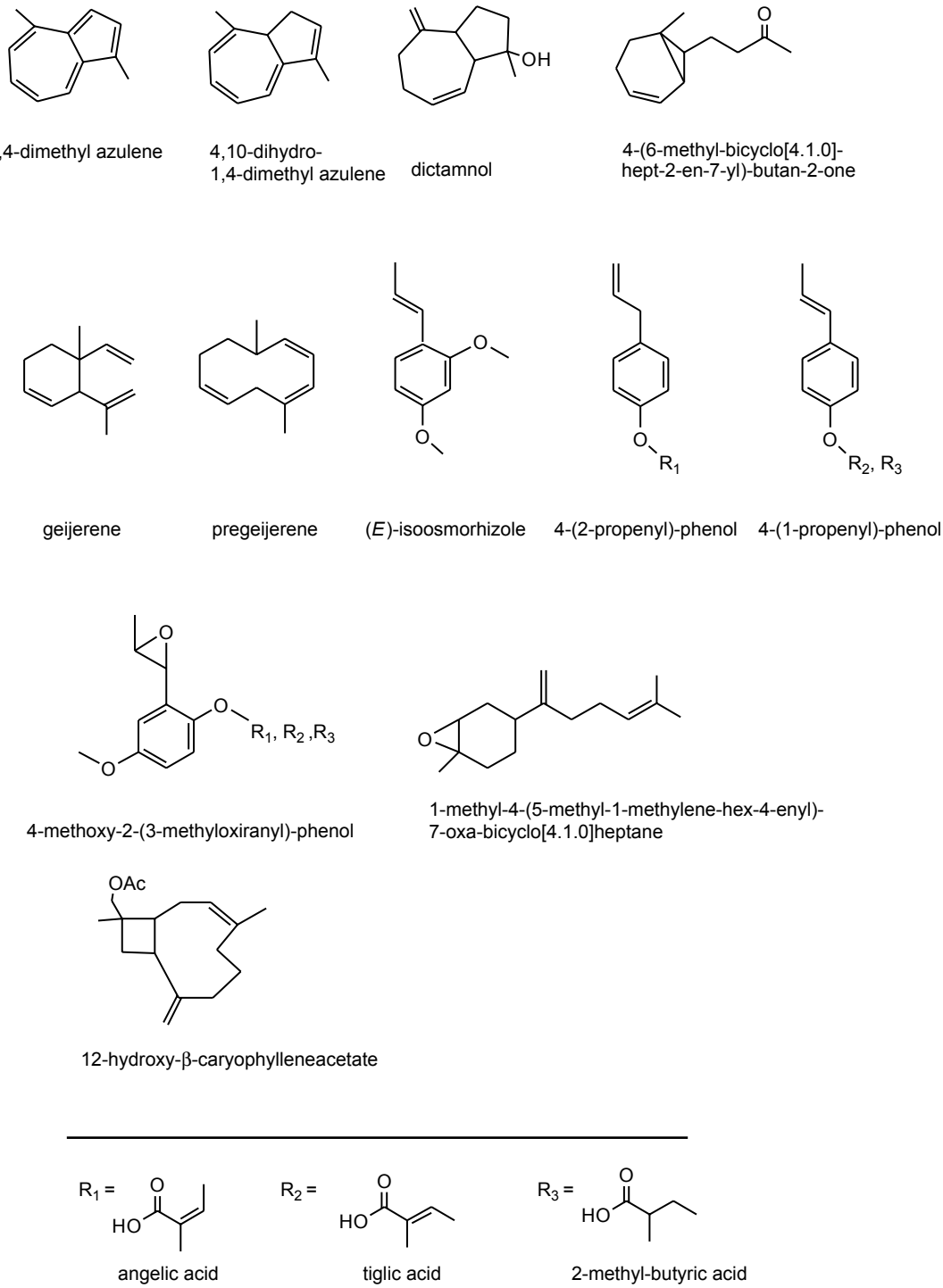

Fig. 1. Representative classes of essential oils of phylogenetically interesting *Pimpinella* species.

materials, locations and collection dates of collection are given in Table 1. Voucher specimens are kept at the Herbarium of the Faculty of Pharmacy of Anadolu University in Eskisehir (ESSE). The choice of *Foeniculum vulgare* for the outgroup was based upon fruit characters, previous systematic analyses (Downie *et al.*, 2001), the availability of essential oil data and chemical composition (major component, anethole). *Foeniculum* is a member of the *Apium* clade in the analysis of Downie *et al.* (2001) and, as such, represents a distinct clade separate from *Pimpinella* within an unresolved Apioid superclade (figure 5 in Downie *et al.*, 2001).

DNA extraction, amplification and sequencing

Total DNA was isolated from *ca* 20 mg of leaf tissue or seed of air-dried plant material or from herbarium specimens using the Qiagen Dneasy Plant Mini Kit, following the manufacturer's protocol. The ITS regions were amplified with the primers ITS 1 (5′-GTCCACTGAACCTTATCATTTAG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′). Double-stranded DNA amplifications were performed in a 20 μl volume containing 2.0 μl of 10 × High Fidelity PCR buffer (Invitrogen), 10.5 μl of dNTP mixture containing 10 mM each nucleotide (final concentration of 0.25 mM each, Invitrogen), 10 pmol/μl (1 μl of 10 pmol/μl stock) each primer, 1 μl of 50 mM $MgSO_4$ (final concentration of 2.5 mM, Invitrogen), 0.2 μl of Platinum Taq High Fidelity (2.0 U/μl, Invitrogen), 2.5 μl (40–50 ng) of template DNA and 10.8 μl of sterile water. The amplification was performed using a thermocycler (PTC 225, MJ Re) that was set to run at 94°C for 1 min for initial denaturation, followed by 30 cycles of 94°C for 30 s to denature the double-stranded template DNA, 57°C for 30 s to anneal primers to single-stranded template DNA, and 72°C for 1 min 30 s to extension. The 30 cycles were followed by a 7 min (72°C) extension and held at 4°C. The PCR products were separated on 1% TAE agarose gels and the band from each reaction was cut out and purified (Qiagen Gel Extraction Kit) for direct sequencing. The psbA-trnH spacer was amplified using the primers psb-F (5′-GTTATGCATGAACGTAATGCTC-3′) and psb-R (trnHR) (5′-CGCGCATGGTGGATTCACAAATC-3′). Amplification reactions for the psbA-trnH region were carried out in a total reaction volume of 25 μl containing 2.5 μl Pfu buffer (Stratagene), 10 mM dNTP (1 μl of 10 mM mixture, Gibco), 10 pmol/μl (2 μl of 10 pmol/μl stock) each primer, 0.5 μl Pfu DNA polymerase (2.5 U/μl unit, Stratagene), 2.5 μl (~50 ng) of template DNA and 14.5 μl of sterile water. PCR protocol was set to run at 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 51°C for 30 s and 72°C for 1 min 30 s. These cycles were followed by an extension for 10 min at 72°C and then held at 4°C. PCR products were run on 1% TAE agarose gel and purified, as above. PCR products were cloned following the instructions of the In Vitrogen TOPO blunt end Cloning Kit. Clones were grown on LB agar containing 100 mg/ml ampicillin and grown overnight. Plasmid DNA was isolated according to the Qiagen miniprep protocol.

Sequencing reactions were conducted using the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). DNA template (50 ng; 0.5 μg of plasmid DNA) was combined with 10 pmol/μl (1.5 μl of 10 pmol/μl stock) of labelled primer with the final volume adjusted to 17 μl. Template/primer mix was mixed with 2 μl each of the Thermo Sequenase Reagent Mix and then the cycle was programmed for 94°C for 1 min followed by 30 cycles at 92°C for 30 s, 60°C for 15 s and 70°C for 15 s, and then held at 4°C. Sequencing reaction products were denatured at 94°C for 2 min and reactions were stopped by the addition of 3 μl of LI-COR stop solution. Sample (1 μl) was applied to each well and run by electrophoresis in 3.75% acrylamide gels. Sequencing of all samples was performed using an NEN Global IR2 DNA Sequencer.

Data analysis

Forward and reverse sequences for each sample were examined using Seqman (DNAStar, Inc.). The aligned sequences were checked and compared using Sequencher 3.0 (Gene Codes Corporation). The manually aligned sequence matrix is available at TreeBase (http://www.treebase.org/treebase). Most of the alignments were clear-cut however, there were length differences that made small regions of the alignment ambiguous. Ambiguous regions were excluded from the analyses. Indels were evaluated and except for one region, were coded separately, particularly when more than one site was involved in the indel.

Sequence data were analysed using parsimony and likelihood techniques as implemented in PAUP*4.0b10 (Swofford, 2001). In all cases, the parsimony analysis involved 100 random starting-point heuristic searches with tree–bisection–reconnection branch swapping (TBR) and zero length branches were collapsed. Bootstrap analyses were also implemented and included 100 replicates of 10 random starting-point heuristic searches.

Each gene region (ITS data and PSB data) was analysed separately to compare topologies and examine congruence. Ultimately, the two data sets were combined (combined data set). Several analysis strategies were executed in a hierarchical manner on each of the gene region data sets and the combined data set. In the first and second strategies, weighting varied. In the first analyses, the transitions and transversions were equally weighted (ti:tv = 1) and indels were excluded from the analysis. In a similar analysis, the indels were recoded as single characters and included in the analysis. In the second strategy analysis, we weighted the transitions and transversions at the inverse of the empirical ti:tv. This was estimated using ML analyses and the Hasegawa–Kishino–Yano (HKY) substitution model (Hasegawa *et al.*, 1985).

Trees obtained from the analyses were saved for comparisons (see Results). The combined analysis trees were

Table 1. Species, collection sites and plant tissues used in this study

Species	Collections sites	Sample material	Date	ESSE No.[a]	GenBank Accession Nos. (ITS/PsbA-trnH)
P. affinis Ledeb.	Turkey: Ordu: Mesudiye	Leaf	June 2001	13890	AY581780/AY587848
P. anisetum Boiss. & Bal.	Turkey: Sivas: Yildizeli	Leaf	July 2001	13937	AY581781/AY587849
P. anisum L.	Turkey: Culture	Fruit	August 2001	14128	AY581782/AY587850
P. antriscoides Boiss. var. *anthriscoides*	Iran: 44 km from Marivan	Leaf		Kew[b]	AY581783/AY587851
P. aromatica Bieb.	Turkey: Artvin: Ardanuc	Fruit		10788	AY581784/AY587852
P. aurea DC.	Turkey: Van: Ercis	Leaf	July 2001	13912	AY581785/AY587853
P. cappadocica Boiss. & Bal. var. *cappadocica*	Turkey: Kayseri: Alidag	Leaf	July 2001	13879	AY581786/AY587854
P. corymbosa Boiss.	Turkey: Siirt: Siirt-Sirnak road	Leaf	July 2001	13899	AY581787/AY587855
P. cretica Poiret var. *arabica* (Boiss.) Boiss.	Israel: 1 km east of village of Taiyba	Leaf		Kew[b]	AY581788/AY587856
P. cretica Poiret var. *cretica*	Turkey: Mugla: 13 km south of Fethiye	Leaf		Kew[b]	AY581789/AY587857
P. eriocarpa Banks & Sol.	Turkey: Urfa: Ceylanpinar	Leaf	May 2001	14127	AY581790/AY587858
P. flabellifolia (Boiss.) Benth. & Hook	Turkey: Sivas: Gurun	Leaf	July 2001	13894	AY581791/AY587859
P. isaurica Matthews	Turkey: Karaman: Ermenek	Leaf	July 2001	13888	AY581792/AY587860
P. kotschyana Boiss.	Turkey: Konya: Eregli	Leaf	July 2001	13886	AY581793/AY587861
P. nudicaulis Trautv.	Turkey: Van: Akcay	Leaf	July 2001	13919	AY581794/AY587862
P. olivieroides Boiss. & Hausskn.	Turkey: Ankara Beynam woods	Leaf	June 2000	13928	AY581795/AY587863
P. paucedentata Matthews	Turkey: Malatya: Akcadag	Leaf	August 2001	14126	AY581796/AY587864
P. peregrina L.	Turkey: Antalya: Akseki	Leaf	June 2001	13877	AY581797/AY587865
P. peucedanifolia Fischer	Turkey: Mus: Solhan	Leaf	July 2001	13913	AY581798/AY587866
P. puberula (DC.) Boiss.	Turkey: Hakkari: Hakkari-Van road	Leaf	July 2001	13909	AY581799/AY587867
P. rhodantha Boiss.	Turkey: Giresun: Tamdere	Leaf	August 2001	13932	AY581800/AY587868
P. saxifraga L.	Turkey: Kars: Sarikamis	Leaf	July 2001	13924	AY581801/AY587869
P. sintenisii Wolff	Turkey: Mardin: Zafran	Leaf		Kew[b]	AY581802/AY587870
P. tragium Vill. ssp. *lithophila*	Turkey: Eskisehir: Turkmen Mountain	Leaf	June 2000	13872	AY581803/AY587871
P. tragium Vill. ssp. *polyclada* (Boiss. & Heldr.)	Turkey: Eskisehir: Sivrihisar	Leaf	June 2001	13891	AY581804/AY587872
P. tragium Vill. ssp. *pseudotragium* (DC.) Matthews	Turkey: Ankara: Kicilcahamam	Leaf	June 2001	13874	AY581805/AY587873
Foeniculum vulgare L.	Turkey: Canakkale	Leaf	August 1984	7520	AY581806/AY587874

[a] ESSE, Herbarium of the Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey.
[b] Supplied by Kew Herbarium, Kew, UK.

examined in MacClade 4.06 (Maddison and Maddison, 2003). The distribution of the isolated oils was examined on the trees (see below). The distribution and putative relationships among the oils within each of the three classes were examined using the concentrated changes test (Maddison and Maddison, 2003) as implemented in MacClade 4.06. The test uses the phylogenetic information to test whether changes among one character are associated with changes in another character. Specifically, given the distribution of one oil (independent variable) on the phylogeny, what is the probability that an alternative oil (dependent variable) will share or not share a similar distribution. The null hypothesis in all cases is that the distribution of the oils is random to one another. Individual oils within two classes of oils were examined, norsesquiterpenes (azulenes, geijerenes) and phenylpropanoids [propenylphenol-type phenylpropanoid (PP) and pseudoisoeugenol-type phenylpropanoid (PSIP)]. It has been hypothesized that the geijerenes are precursors to the azulenes (Kubeczka *et al.*, 1989). Similarly, anethole has been proposed to be the precursor to the many PSIPs (Reichling *et al.*, 1995). In all examinations, taxa that were not chemically sampled were excluded from the analyses.

Isolation of the essential oil

Dried fruits, aerial parts (without fruits) and roots of *Pimpinella* species were hydro-distilled for 3 h using a Clevenger-type apparatus to obtain essential oils. There were unsatisfactory sample quantities of oils for *P. antriscoides* var. *antriscoides*, *P. cretica* var. *arabica* and var. *cretica*, *P. eriocarpa*, *P. paucidentata* and *P. sintenisii*. Oils from those species could not be obtained due to insufficient sample quantities.

Chromatographic analysis

The essential oils were analysed by gas chromatography using a Hewlett Packard 6890 system. An HP-Innowax FSC (60 m × 0.25 mm i.d., with 0.25 μm film thickness) was used with nitrogen as the carrier gas (1 ml/min). The oven temperature was kept at 60°C for 10 min, programmed to 220°C at a rate of 4°C/min, then kept constant at 220°C for 10 min, and then programmed to 240°C at a rate of 1°C/min. The injector temperature was at 250°C. Relative percentages (i.e. percentage peak area relative to total peak area) were obtained from electronic integration measurements using a flame ionization detector (250°C). *n*-Alkanes were used as reference point in the calculation of the relative retention indices.

Identification of compounds

The samples were analysed by gas chromatography–mass spectrometry (GC-MS) employing the same chromatographic conditions as described above, using a Hewlett Packard GCD system with helium as carrier gas (1 ml/min). The split ratio was adjusted at 50:1. MS was recorded at 70 eV. The mass range was from *m/z* 35 to 425. Library search was carried out using the Wiley GC-MS Library, Mass Finder Library and the in-house Baser Library of Essential Constituents (Tables 2–4). Compounds are listed based on their retention index.

Results

Phylogenetic analyses

Lengths of the aligned sequences are 638 bp for ITS and *ca* 465 for psbA-trnH spacer. Two indels were each coded as a single character for the ITS data, one insertion (3 nt) and one deletion (1 nt) event: three sites were excluded due to ambiguity. Four indels were coded in the psbA data, one deletion (4 bp) and three insertions (12 bp, 3 bp, 1 bp): a total of 45 sites were excluded due to ambiguity. The ITS sequence data had a GC content of *ca* 54% including the outgroup. A total of 88 sites and two indels were phylogenetically informative and resulted in 18 trees (305 steps, r.c.i. = 0.5246, r.i. = 0.67). The PSB sequence data had a lower GC content at *ca* 35%, typical of cpDNA. A total of 22 characters plus four indels were phylogenetically informative and the analyses resulted in over 10,000 trees (10,000 trees maximum, 115 steps, r.c.i. = 0.74, r.i. = 0.833). Overall, the topologies of the ITS trees were present within the PSB trees and the strict consensus trees of each of the gene regions were congruent.

The combined analysis included 1103 sites (48 ambiguous sites excluded). A total of 110 characters were parsimony informative and the six indels (total of 24 sites) were coded in the final analysis (746 sites were constant, 175 were not parsimony informative). The topologies from the different analysis strategies did not differ although there was variation in branch support for some of the taxa or clades. A total of 12 trees resulted from the combined analysis (187 steps, r.c.i. = 0.561, r.i. = 0.696) using the indels. ML analysis using the HKY substitution model on the data produced a tree with a length of $-\ln L = 3918.3$ and was similar to the strict consensus and bootstrap consensus tree (Fig. 2; it should be noted that in the strict consensus, *P. paucidentata* is unresolved in a trichotomy with the *P. cretica* clade and *P. puberula*).

Table 2. Composition of fruit essential oils of *Pimpinella* species

#	RRI	Compounds	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	1032	α-Pinene	–	–	–	<5.0	<5.0	<5.0	<5.0	–	tr	<5.0	tr	tr	tr	tr	–	<5.0	<5.0	<5.0	15.7
2	1100	Undecane	–	–	–	–	–	–	–	–	–	<5.0	–	–	76.5	–	–	–	–	–	–
3	1118	β-Pinene	–	–	–	<5.0	<5.0	<5.0	<5.0	–	<5.0	<5.0	–	–	<5.0	tr	<5.0	20.6	<5.0	tr	<5.0
4	1132	Sabinene	–	–	–	<5.0	5.7	tr	tr	–	tr	<5.0	–	–	–	5.4	–	40.7	6.3	tr	<5.0
5	1174	Myrcene	–	–	–	<5.0	<5.0	<5.0	–	–	tr	<5.0	–	–	<5.0	<5.0	–	14.1	<5.0	<5.0	<5.0
6	1203	Limonene	<5.0	–	–	<5.0	<5.0	<5.0	28.0	<5.0	<5.0	<5.0	tr	–	tr	63.4	tr	<5.0	<5.0	tr	<5.0
9	1304	Isogeijerene	7.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	1337	Geijerene	58.7	tr	–	<5.0	–	–	–	–	–	–	–	tr	<5.0	<5.0	<5.0	tr	22.9	–	–
12	1594	Pregeijerene	19.5	–	–	–	–	–		tr	tr	–	–	–	<5.0	<5.0	–	–	5.0	–	–
13	1594	*trans*-β-Bergamotene	–	–	–	–	–	–	–	–	–	–	–	70.3	–	–	–	–	–	–	–
14	1612	β-Caryophyllene	<5.0	–	–	<5.0	8.5	33.2		5.1	49.3	<5.0	14.2	<5.0	<5.0	<5.0	<5.0	–	<5.0	–	<5.0
15	1668	(*Z*)-β-Farnesene	–	–	–	<5.0	<5.0	<5.0	–	7.2	–	–	tr	–	tr	tr	34.6	<5.0	<5.0	57.3	–
16	1687	α-Humulene	–	–	–	–	–	7.1	–	<5.0	11.0	–	<5.0	<5.0	<5.0	–	–	<5.0	–	–	<5.0
17	1687	Methyl chavicol	–	16.4	<5.0	–	–	–	–	–	–	tr	–	–	–	–	–	–	–	–	–
18	1711	γ-Himachalene	–	–	<5.0	–	9.3	<5.0	–	–	<5.0	–	<5.0	–	<5.0	–	8.8	–	–	–	12.4
19	1726	Germacrene D	tr	–	–	5.7	–	9.1	–	8.3	<5.0	<5.0	<5.0	–	<5.0	tr	–	<5.0	<5.0	<5.0	–
20	1726	α-Zingiberene	–	–	<5.0	–	–	–	–	15.7	–	tr	<5.0	–	<5.0	–	<5.0	<5.0	–	–	8.3
22	1741	β-Bisabolene	–	tr	<5.0	33.1	–	<5.0	–	<5.0	–	–	<5.0	<5.0	–	–	32.8	<5.0	29.8	–	18.8
23	1755	Bicyclogermacrene	–	–	–	<5.0	12.0	<5.0	–	<5.0	<5.0	<5.0	tr	<5.0	5.0	–	–	tr	<5.0	–	–
26	1845	(*E*)-Anethole	–	80.7	94.2	tr	<5.0	tr	63.6	–	–	63.5	–	<5.0	–	–	tr	–	tr	–	<5.0
29	2030	Methyl eugenol	–	–	–	–	–	–	–	–	–	–	70.6	–	–	23.1	<5.0	–	–	–	–
30	2038	1-Methyl-4-(5-methyl-1-methylene-hex-4-enyl)-7-oxa-bicyclo [4.1.0] heptane	–	–	–	33.5	–	–	–	–	–	–	<5.0	–	–	–	–	–	–	–	–
33	2182	4-(1-Propenyl)-phenyl isobutyrate	–	–	–	–	–	–	–	–	<5.0	–	–	–	<5.0	–	–	–	–	–	–
34	2212	*trans*-Isoosmorhizole	–	–	–	–	–	–	–	–	–	20.6	–	tr	–	–	–	–	–	–	–
35	2252	4-(2-Propenyl)-phenyl angelate	–	–	–	–	–	–	–	13.7	–	–	–	–	–	–	–	–	–	–	–
37	2284	4-(1-Propenyl)-phenyl-2-methyl butyrate	–	–	–	<5.0	<5.0	–	–	–	tr	–	<5.0	–	<5.0	–	<5.0	<5.0	<5.0	–	<5.0
38	2289	*cis*-Isoelemicine	–	–	–	–	–	–	–	–	–	–	5.3	–	–	–	–	–	–	–	–
40	2331	12-Hydroxy-β-caryophyllene acetate	–	–	–	–	–	<5.0	–	–	11.5	–	–	–	–	–	–	–	–	–	–
41	2353	Chavicol	–	–	–	–	–	–	–	<5.0	–	–	–	–	–	–	–	–	–	–	–
42	2406	4-(1-Propenyl)-phenyl tiglate	–	–	–	<5.0	–	–	–	<5.0	–	–	–	–	–	–	–	–	–	–	<5.0
44	2506	4-(3-Methyloxiranyl)-phenyl-2-methyl butyrate	–	–	–	<5.0	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–	–
45	2567	Pseudoisoeugenyl-2-methyl butyrate	–	–	<5.0	–	<5.0	–	–	–	–	–	–	–	–	–	–	–	tr	–	–

Table 2. *Continued*

#	RRI	Compounds	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
46	2613	4-Methoxy-2-(3-methyl oxiranyl)-phenyl isobutyrate	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–	–	–	–	–	–
47	2642	4-(3-Methyloxiranyl)-phenyl tiglate	–	–	–	<5.0	–	–	–	–	–	–	–		–	–	–	–	–	–	–
49	2698	Epoxypseudoisoeugenyl-2 methyl butyrate	–	–	–	–	tr	–	–	–	–	–	tr	<5.0	–	–	–	<5.0	<5.0	20.0	10.0
50	2766	4-Methoxy-2-(1-propenyl)-phenyl tiglate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–	–	–
51	2825	4-Methoxy-2-(3-methyl oxiranyl)-phenyl angelate	–	–	–	–	–	–	–	tr	–	–	–	–	–	–	<5.0	–	–	<5.0	<5.0
52	2926	4-Methoxy-2-(3-methyl oxiranyl)-phenyl tiglate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–	5.7	–

RRI, relative retention indices calculated against *n*-alkanes; tr, trace (≤0.1%).

A, *P. affinis*; B, *P. anisetum*; C, *P. anisum*; D, *P. aurea*; E, *P. cappacodica* var. *cappadocica*; F, *P. corymbosa*; G, *P. flabellifolia*; H, *P. isaurica*; I, *P. kotschyana*; J, *P. nudicaulis*; K, *P. olivieroides*; L, *P. peregrina*; M, *P. peucedanifolia*; N, *P. pubberula*; O, *P. rhodanta*; P, *P. saxifraga*; Q, *P. tragium* ssp. *lithophila*; R, *P. tragium* ssp. *polyclada*; S, *P. tragium* ssp. *pseudotragium*.

Oil constituent distribution of phylogeny

Two major classes of oils, the norsesquiterpenes (geijerenes and azulenes) and the phenylpropanoids, are given in Tables 2–4. The relationships among the isolates in each of these groups and their relationships to one another were examined using the concentrated changes test. The concentrated changes test requires fully resolved dichotomous trees. The analyses were performed on four trees. These four trees were chosen because there were slight differences in some of the patterns of chemical distribution among them. In all cases, there was no significant difference in the statistical associations of the reported chemicals among the four different analyses. The distribution of the norsesquiterpenes (geijerenes and azulene) is illustrated on the topology in Fig. 3. The norsesquiterpenes, the geijerenes (**9**, **10**, **12**) and azulenes (**25**, **27**, **31**, **32**, **39**), share similar patterns of distribution overall and their distribution is non-random to one another. The probability that the azulenes are randomly distributed relative to the geijerenes is 0.00 038 and, conversely, the probability that the geijerenes are randomly distributed relative to the azulenes is 0.00 045. The distributions of the azulenes and geijerenes to one another are significantly non-random to one another. Within the geijerenes, all but two of them (**9** and **10**) show a non-random distribution relative to one another (Table 5). Within the azulenes most of them show a non-random distribution relative to another.

The results from the concentrated changes test among the different phenylpropanoids on the tree are given in Fig. 4 and the chemical relationships are depicted in Table 6. Anethole, the putative precursor to pseudoisoeugenol-type (PSIP) phenylpropanoids, is present in all sampled taxa. However, there was significant variation among the taxa regarding the concentration of anethole and the presence of putative propenylphenol derivatives (PPs). In many taxa, the anethole concentration was significantly higher (>63.0%) than in other taxa (marked 'high' and 'low', respectively, in Fig. 4). In those taxa with low anethole concentrations there tends to be multiple different PPs and PSIPs whereas taxa with high concentrations of anethole showed little or no presence of other PSIPs (the exception to this is *P. anisetum*).

In several cases, there is a significant association between different compounds and their distribution on the tree. In particular, there appear to be three tightly associated groups of PP and PSIP compounds (**33**, **37**, **44**, **47**, **45**, **49** = group 1; **42**, **48**, **50**, **51**, **52** = group 2; **43**, **46** = group 3). Within each group, the presence of a single compound is significantly associated with the distribution of all other chemicals in the group (group 1, **45**; group 2, **48**).

Table 3. Composition of aerial parts (without fruits) essential oils of *Pimpinella* species

#	RRI	Compounds	A	B	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	1032	α-Pinene	–	–	12.1	<5.0	<5.0	5.1	–	<5.0	16.5	tr	<5.0	<5.0	tr	<5.0	5.9	tr	12.2	30.5
2	1100	Undecane	–	–	–	–	–	–	–	–	<5.0	–	–	65.1	–	–	–	–	–	–
3	1118	β-Pinene	–	–	8.0	<5.0	<5.0	<5.0	–	<5.0	14.7	tr	–	tr	tr	tr	28.4	<5.0	<5.0	<5.0
4	1132	Sabinene	–	–	20.7	17.0	tr	–	–	<5.0	10.7	–	–	<5.0	<5.0	tr	14.3	<5.0	<5.0	<5.0
5	1174	Myrcene	–	–	<5.0	<5.0	<5.0	–	–	<5.0	<5.0	–	–	–	<5.0	tr	18.9	tr	<5.0	<5.0
6	1203	Limonene	<5.0	–	<5.0	<5.0	tr	34.2	–	<5.0	<5.0	tr	–	tr	36.5	<5.0	11.1	<5.0	<5.0	<5.0
10	1337	Geijerene	39.6	<5.0	tr	–	tr	–	<5.0	tr	–	tr	–	<5.0	7.3	<5.0	tr	32.2	–	<5.0
11	1455	Clavukerin B	8.6	–	–	–	–	–	–	–	–	–	–	tr	–	tr	–	8.3	–	–
12	1591	Pregeijerene	10.6	–	–	–	–	–	tr	tr	–	–	–	tr	<5.0	–	–	8.3	–	tr
13	1594	*trans*-β-Bergamotene	–	–	–	–	–	–	–	–	–	–	40.9	–	–	–	–	–	–	–
14	1612	β-Caryophyllene	<5.0	–	<5.0	<5.0	32.5	–	<5.0	39.2	<5.0	<5.0	5.1	<5.0	<5.0	–	<5.0	<5.0	–	<5.0
15	1668	(*Z*)-β-Farnesene	–	–	<5.0	<5.0	<5.0	–	<5.0	tr	–	–	–	–	–	–	<5.0	–	22.5	tr
16	1687	α-Humulene	<5.0	–	–	<5.0	7.3	–	<5.0	8.7	–	<5.0	<5.0	tr	tr	–	<5.0	–	–	–
17	1687	Methyl chavicol	–	13.2	–	–	–	–	tr	–	<5.0	–	–	–	–	–	–	tr	–	tr
19	1726	Germacrene D	tr	–	<5.0	6.1	11.6	–	<5.0	5.4	<5.0	<5.0	–	<5.0	<5.0	16.5	<5.0	tr	–	–
20	1726	α-Zingiberene	–	–	–	–	–	–	<5.0	–	<5.0	–	–	<5.0	–	<5.0	–	tr	<5.0	<5.0
21	2406	4-(1-Propenyl)-phenyl tiglate	–	–	<5.0	–	–	–	12.6	–	–	–	–	–	–	–	–	–	–	–
22	1741	β-Bisabolene	–	tr	9.6	–	–	–	<5.0	–	–	>5.0	<5.0	<5.0	–	11.5	<5.0	<5.0	–	5.7
26	1845	(*E*)-Anethole	tr	54.2	<5.0	<5.0	–	41.0	<5.0	–	27.8	–	–	<5.0	–	<5.0	tr	tr	tr	tr
27	1740	β-Himachalene	–	–	–	–	–	–	–	–	–	–	–	–	–	16.5	–	–	–	–
27	1881	4-(6-Methyl-bicyclo[4.1.0]-hept-2-en-7-yl)-butan-2-one	<5.0	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–	<5.0	–	–
28	2008	Caryophyllene oxide	<5.0	–	<5.0	<5.0	<5.0	–	–	<5.0	–	5.6	–	–	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
29	2030	Methyl eugenol	–	–	–	–	–	tr	–	–	–	51.6	–	–	29.6	–	–	–	–	–
30	2038	1-Methyl-4-(5-methyl-1-methylene-hex-4-enyl)-7-oxa-bicyclo [4.1.0] heptane	–	–	19.8	–	–	–	–	–	–	–	<5.0	–	–	–	–	–	–	–
32	2170	Dictamnol	<5.0	–	–	–	–	–	–	tr	–	–	–	<5.0	<5.0	<5.0	–	6.1	–	–
33	2182	4-(1-Propenyl)-phenyl isobutyrate	–	–	–	–	–	–	–	<5.0	–	–	–	tr	–	–	–	–	–	–
34	2212	*trans*-Isoosmorhizole	–	–	–	–	–	–	–	–	12.2	–	<5.0	–	–	–	–	–	–	–
35	2252	4-(2-Propenyl)-phenyl angelate	–	–	–	–	–	–	43.3	–	–	–	–	–	–	–	–	–	–	–
36	2255	β-Eudesmol	–	–	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–	–	–
37	2284	4-(1-Propenyl)-phenyl-2-methylbutytate	–	–	<5.0	<5.0	<5.0	–	–	<5.0	–	tr	–	<5.0	–	<5.0	<5.0	–	–	<5.0
38	2289	*cis*-isoelemicine	–	–	–	–	–	–	–	–	–	5.0	–	–	–	–	–	–	–	–
39	2291	1,4-Dimethylazulene	<5.0	–	–	<5.0	–	–	–	–	–	–	–	–	>5.0	<5.0	–	<5.0	–	–
40	2331	12-Hydroxy-β-caryophyllene acetate	–	–	–	–	–	–	–	–	<5.0	–	–	–	–	–	–	–	–	<5.0
41	2353	Chavicol	–	–	–	–	–	–	<5.0	–	–	–	–	–	–	–	–	–	–	–
44	2506	4-(3-Methyloxiranyl)-phenyl-2-methyl butyrate	–	–	<5.0	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–	

Table 3. *Continued*

#	RRI	Compounds	A	B	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
46	2613	4-Methoxy-2-(3-methyloxiranyl)-phenyl isobutyrate	–	–	–	–	–	–	–	–	–	–	5.5	–	–	–	–	–	–	–
47	2642	4-(3-Methyloxiranyl)-phenyl tiglate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
48	2658	4-Methoxy-2-(1-propenyl)-phenyl angelate	–	–	–	–	–	–	<5.0	–	–	–	–	–	–	–	–	–	–	<5.0
49	2698	Epoxypseudoisoeugenyl-2 methyl butyrate	<5.0	23.6	–	<5.0	tr	–	–	–	–	–	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	21.7	5.7
50	2766	4-Methoxy-2-(1-propenyl)-phenyl tiglate	–	–	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–	<5.0	–
51	2825	4-Methoxy-2-(3-methyloxiranyl)-phenyl angelate	<0.5	5.1	–	–	–	–	<5.0	–	–	–	8.1	–	–	<5.0	–	–	<5.0	<5.0
52	2926	4-Methoxy-2-(3-methyloxiranyl)-phenyl tiglate	–	–	–	–	–	–	<5.0	–	–	–	<5.0	–	–	5.9	–	–	<5.0	–

RRI, relative retention indices calculated against *n*-alkanes; tr, trace (≤0.1%).

A, *P. affinis*; B, *P. anisetum*; C, *P. anisum*; D, *P. aurea*; E, *P. cappacodica* var. *cappadocica*; F, *P. corymbosa*; G, *P. flabellifolia*; H, *P. isaurica*; I, *P. kotschyana*; J, *P. nudicaulis*; K, *P. olivieroides*; L, *P. peregrina*; M, *P. peucedanifolia*; N, *P. puberula*; O, *P. rhodanta*; P, *P. saxifraga*; Q, *P. tragium* ssp. *lithophila*; R, *P. tragium* ssp. *polyclada*; S, *P. tragium* ssp. *pseudotragium*.

Table 4. Composition of root essential oils of *Pimpinella* species

#	RRI	Compounds	A	B	D	E	F	G	H	I	J	K	L	M	O	P	Q	R	S
6	1203	Limonene	<5.0	–	<5.0	–	–	17.2	–	tr	tr	–	–	–	<5.0	<5.0	tr	–	tr
7	1255	γ-Terpinene	<5.0	–	<5.0	–	–	5.0	17.3	tr	<5.0	–	–	–	–	tr	<5.0	<5.0	tr
8	1280	*p*-Cymene	<5.0	–	tr	–	–	–	8.4	tr	<5.0	–	–	–	tr	–	<5.0	<5.0	<5.0
9	1304	Isogeijerene	<5.0	–	–	<5.0	<5.0	–	–	–	–	–	–	–	–	–	<5.0	–	–
10	1337	Geijerene	35.7	<5.0	tr	<5.0	<5.0	–	5.4	7.3	–	<5.0	tr	<5.0	<5.0	<5.0	26.7	<5.0	<5.0
11	1455	Clavukerin B	<5.0	–	–	<5.0	–	–	<5.0	–	–	<5.0	–	–	–	–	–	–	–
12	1591	Pregeijerene	8.8	<5.0	–	–	<5.0	–	<5.0	<5.0	–	–	–	tr	<5.0	tr	6.7	<5.0	tr
22	1741	β-Bisabolene	–	<5.0	6.1	–	<5.0	–	–	<5.0	–	<5.0	<5.0	–	–	tr	<5.0	–	<5.0
24	1783	β-Sesquiphellandrene	–	–	–	–	–	–	–	–	–	–	9.5	–	–	–	–	–	–
25	1815	4,10-Dihydro-1,4-dimethylazulene	<5.0	<5.0	–	7.1	<5.0	–	–	–	–	5.5	–	<5.0	–	–	13.5	–	–
26	1845	(*E*)-Anethole	<5.0	5.1	<5.0	<5.0	–	67.9	tr	–	12.5	–	<5.0	tr	<5.0	–	–	–	<5.0
27	1881	4-(6-Methyl-bicyclo[4.1.0]hept-2-en-7-yl)-butan-2-one	<5.0	–	–	tr	–	–	–	–	–	–	–	–	tr	–	<5.0	–	<5.0
30	2038	1-Methyl-4-(5-methyl-1-methylene-hex-4-enyl)-7-oxa-bicyclo [4.1.0] heptane	–	–	9.7	–	–	–	–	–	–	–	<5.0	–	–	–	–	–	–
31	2152	8-Epi-dictamnol	<5.0	–	–	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–
32	2170	Dictamnol	<5.0	<5.0	–	–	–	–	–	–	–	–	–	–	<5.0	<5.0	<5.0	–	<5.0
33	2182	4-(1-Propenyl)-phenyl isobutyrate	–	–	–	–	<5.0	–	–	<5.0	–	–	–	–	–	–	–	–	–
34	2212	*trans*-Isoosmorhizole	–	–	–	–	–	<5.0	–	–	78.9	–	tr	–	–	–	–	–	<5.0
35	2252	4-(2-Propenyl)-phenyl angelate	–	–	–	–	–	–	10.8	–	–	–	–	–	–	–	–	–	–
36	2255	β-Eudesmol	–	–	–	–	–	–	–	–	–	–	–	–	8.7	–	–	–	–
37	2284	4-(1-Propenyl)-phenyl-2-methyl butyrate	–	<5.0	<5.0	19.1	33.8	–	–	34.3	–	39.0	tr	5.6	–	<5.0	–	–	–
39	2291	1,4-Dimethylazulene	<5.0	<5.0	–	<5.0	<5.0	–	–	–	–	<5.0	–	–	tr	–	<5.0	–	–
41	2353	Chavicol	–	–	–	tr	–	–	<5.0	–	–	–	–	–	–	–	–	–	–
42	2406	4-(1-Propenyl)-phenyl tiglate	–	–	<5.0	tr	–	–	16.1	–	–	–	–	–	–	–	–	–	<5.0
43	2462	4-Methoxy-2-(1-propenyl)-phenyl isobutyrate	–	–	–	–	–	–	–	–	–	–	tr	tr	–	–	–	–	–
44	2506	4-(3-Methyloxiranyl)-phenyl-2-methyl butyrate	–	–	<5.0	–	–	–	–	–	–	–	<5.0	<5.0	–	<5.0	–	–	–
45	2567	Pseudoisoeugenyl-2-methyl butyrate	–	<5.0	<5.0	<5.0	–	–	–	<5.0	–	<5.0	<5.0	<5.0	–	<5.0	–	–	<5.0
46	2613	4-Methoxy-2-(3-methyloxiranyl)-phenyl isobutyrate	–	–	–	–	–	–	–	–	–	–	44.8	<5.0	–	–	–	–	–
48	2658	4-Methoxy-2-(1-propenyl)-phenyl angelate	–	–	–	–	–	–	<5.0	–	–	–	–	–	<5.0	–	–	–	<5.0
49	2698	Epoxypseudoisoeugenyl-2-methyl butyrate	<5.0	56.4	39.0	43.0	42.8	–	–	35.5	–	32.6	26.8	82.6	<5.0	66.6	<5.0	16.0	18.6
50	2766	4-Methoxy-2-(1-propenyl)-phenyl tiglate	–	–	–	–	–	–	–	–	–	–	–	–	<5.0	–	–	<5.0	–
51	2825	4-Methoxy-2-(3-methyloxiranyl)-phenyl angelate	11.2	8.0	–	<5.0	–	–	6.8	–	–	–	tr	–	29.1	–	–	39.9	30.7
52	2926	4-Methoxy-2-(3-methyloxiranyl)-phenyl tiglate	tr	<5.0	<5.0	<5.0	–	–	<5.0	–	–	–	<5.0	–	29.1	–	–	12.1	<5.0

RRI, relative retention indices calculated against *n*-alkanes; tr, trace (≤0.1%).

A, *P. affinis*; B, *P. anisetum*; C, *P. anisum*; D, *P. aurea*; E, *P. cappacodica* var. *cappadocica*; F, *P. corymbosa*; G, *P. flabellifolia*; H, *P. isaurica*; I, *P. kotschyana*; J, *P. nudicaulis*; K, *P. olivieroides*; L, *P. peregrina*; M, *P. peucedanifolia*; N, *P. puberula*; O, *P. rhodanta*; P, *P. saxifraga*; Q, *P. tragium* ssp. *lithophila*; R, *P. tragium* ssp. *polyclada*; S, *P. tragium* ssp. *pseudotragium*.

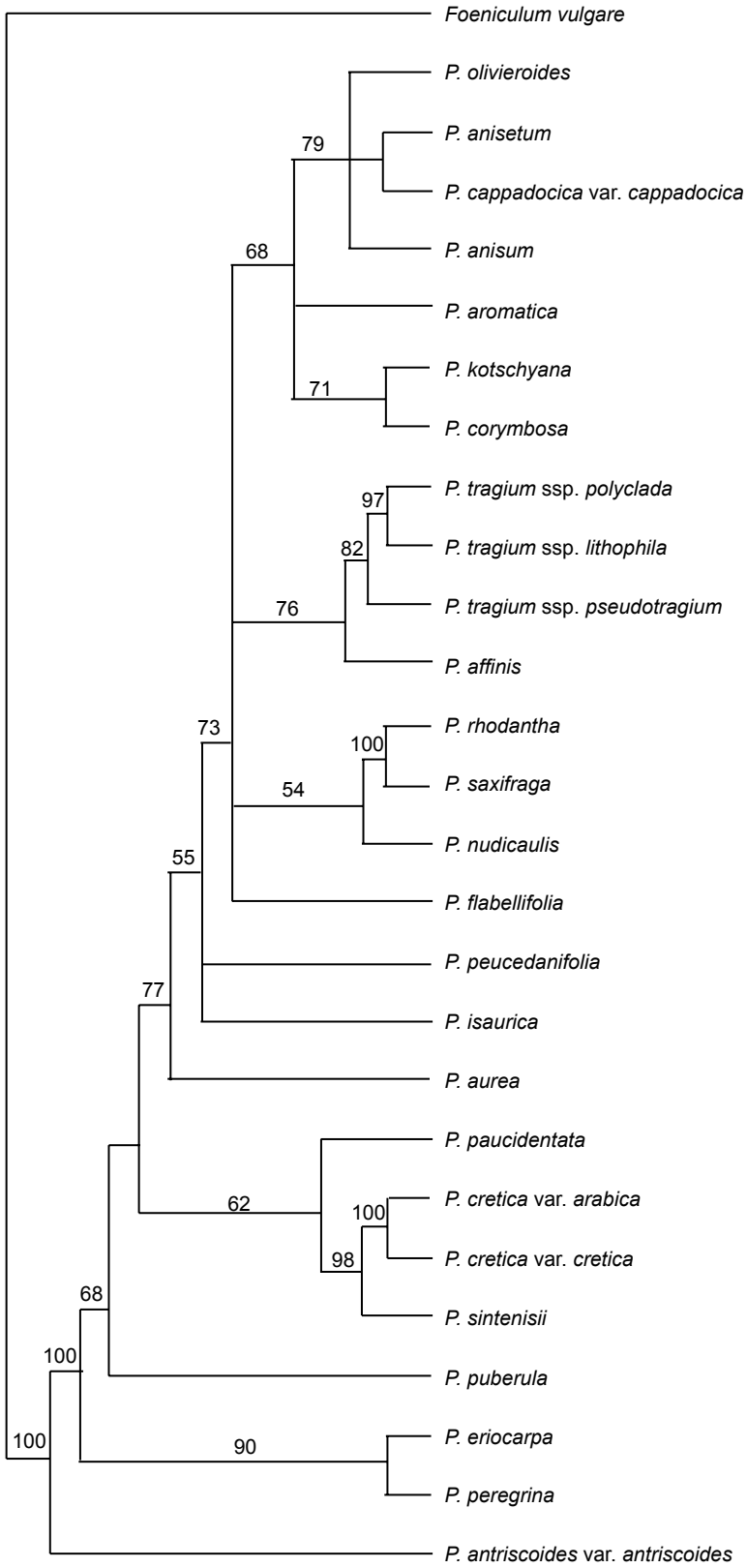

Fig. 2. Bootstrap consensus cladogram depicting relationships among *Pimpinella* species based on ITS and PsbA spacer data. Numbers on branches represent bootstrap support. *Foeniculum vulgare* was used as the outgroup taxon.

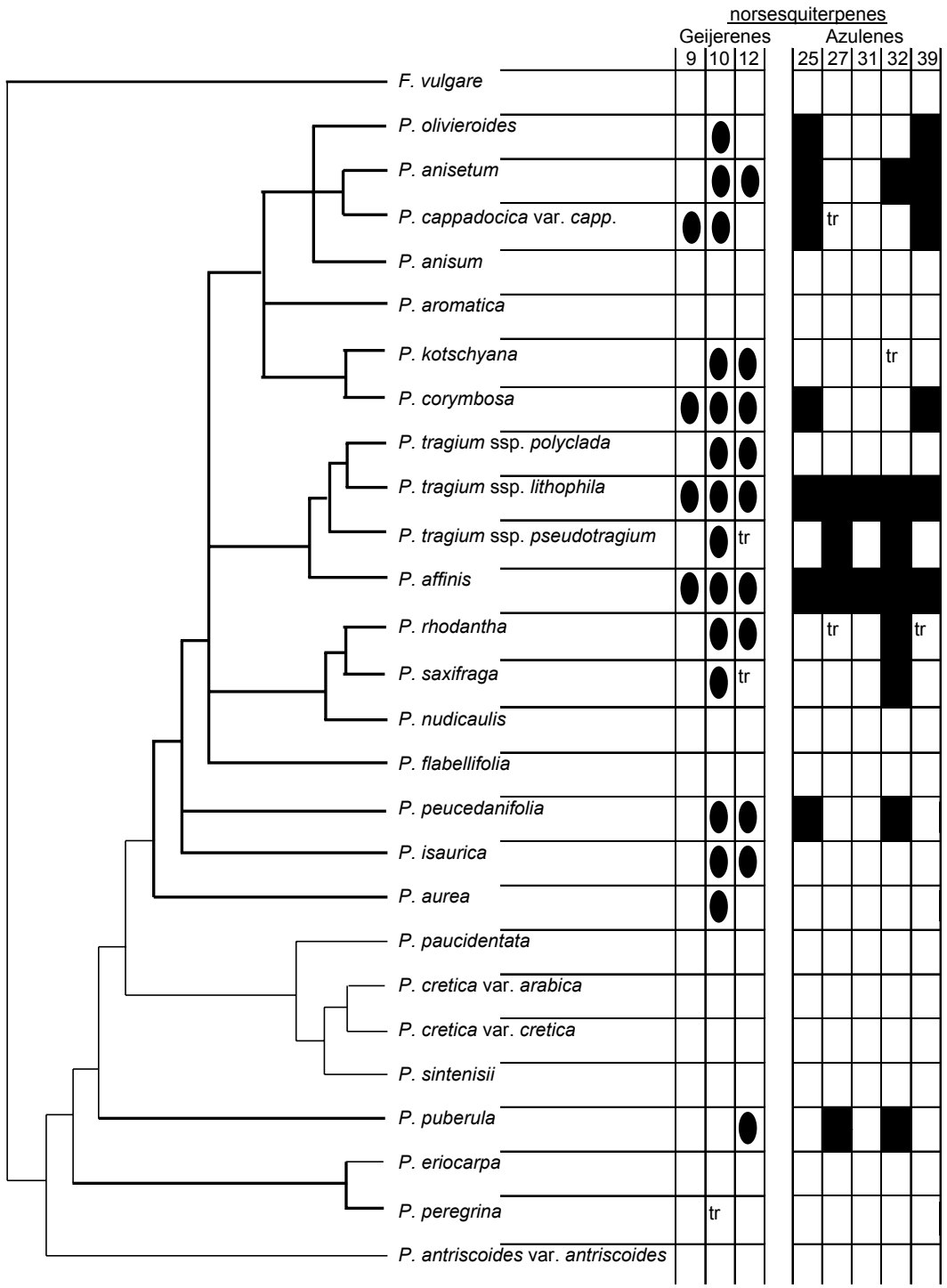

Fig. 3. Distributional relationship among geijerene and azulene derivatives. The presence of geijerenes are denoted by ovals; the presence of azulenes are denoted by rectangles. Taxa in clades with thin branches denote taxa that were not chemically sampled. Trace (tr) ≤ 0.1%. The geijerene and azulene numbers represent the compounds in Tables 2–4.

Table 5. Statistics of azulene and geijerenes

	9	**10**	**12**	**25**	**27**	**31**	**32**	**39**
9	xxx	0.042	0.027	0.0022	0.0019	0.0079	0.0022	0.012
10	0.058	xxx	0.0003	0.002	0.058	0.015	0.058	0.0006
12	0.037	0.0006	xxx	0.05	0.037	0.0098	0.042	0.009
25	0.025	0.060	0.019	xxx	0.025	0.111	0.042	0.00 092
27	0.0019	0.040	0.020	0.002	xxx	0.0079	0.0022	0.008
31	0.0067	0.002	0.004	0.030	0.0067	xxx	0.0067	0.037
32	0.0022	0.048	0.014	0.014	0.0022	0.0079	xxx	0.014
39	0.037	0.015	0.019	0.0004	0.042	0.111	0.037	xxx

The numbers in bold represent azulene and geijerene derivatives found in *Pimpinella* species reported in Tables 2–4.

Discussion

Phylogenetic comparisons of chemical profiles

It is assumed that the distribution of chemical components is often shared among close relatives. An independently derived phylogenetic analysis can thus provide insights into identifying taxa that could have important chemical profiles. In this study, we found that the distribution of essential oils among the different species of *Pimpinella* is, in some cases, shared among closely related species but in many cases is not. Instead, we found that there are novel patterns of association and non-association among the essential oils within each of the different classes examined. The distribution of the different classes of compounds and their associations with one another, given our knowledge regarding their biosynthetic pathways, indicates that this information in conjunction with the phylogeny provides valuable information regarding the presence and perhaps evolution of the different classes of compounds.

The endemic taxa, *P. cappadocica* var. *cappadocica* and *P. anisetum*, are supported as a monophyletic group in spite of different chemical profiles. Morphologically, these species are similar. The differences between them are the leaf segment (ovate or linear) and petals (hairy or glabrous). Essential oils obtained from fruits and aerial parts (without fruits) of *P. anisetum* contain (*E*)-anethole (**26**) (80.7 and 54.2%, respectively) as the major component. In contrast to fruit oil, the essential oil aerial parts (without fruits) and root oil contain primarily epoxypseudoisoeugenyl-2-methyl butyrate (**49**) (23.6 and 56.4%, respectively) (Tables 2–4). Bicyclogermacrene (12.0%) in the fruit oil, sabinene (17.0%) (**4**) in the (without fruits) oil and the 4-(1-propenyl)-phenyl-2-methyl butyrate (19.1%) (**37**) in the root oil are major components for *P. cappadocica* var. *cappadocica* (Tables 2 and 4). The root oil has moderate amounts of 4,10-dihydro-dimethylazulene (7.1%) and 1,4-dimethylazulene (<5.0%) along with unique pseudoisoeugenol-type phenylpropanoids (Table 4).

Although there are no obvious morphological characters supporting a close relationship between *P. anisum* and *P. olivieroides*, the molecular data support a relationship of these two taxa with *P. cappadocica* and *P. anisetum*. The fruits of both *P. anisum* and *P. olivieroides* are rich in phenylpropanoids, (*E*)-anethole (94.2%) (**26**) and methyl eugenol (70.6%), respectively. The composition of the root essential oil of *P. olivieroides* is generally dominated by 4-(1-propenyl)-phenyl-2-methyl butyrate (39.0%) (**37**) and epoxypseudoisoeugenyl 2-methylbutyrate (32.6%) (**49**) in contrast to fruit and aerial parts (without fruits) oils (Table 4).

According to chemical profile and molecular data, *P. corymbosa* and *P. kotschyana* are closely related to each other. But morphologically these species are different from one another, there being differences in leaf segment length, umbel and fruit structure, and pubescence. GC-MS data showed that the chemical profiles of *P. corymbosa* and *P. kotschyana* are similar (Tables 2–4). The most significant feature in these species is the considerable amount of β-caryophyllene (32.2 and 49.3%, respectively) (Tables 2 and 3). The analyses of the essential fruit oil of these species revealed the presence of β-caryophyllene-type oxygenated sesquiterpene. The structure of this constituent was established as 12-hydroxy-β-caryophyllene acetate (11.5%) (**40**) by means of spectral data (Fig. 1) (Tabanca *et al.*, 2005).

P. aromatica has a completely different chemical fingerprint to *P. corymbosa* and *P. kotschyana*, and are a weakly supported monophyletic group. The main compounds of fruit oil are characterized as methyl chavicol (92.0%) and (*E*)-anethole (7.2%) (Baser *et al.*, 1996). The essential oils of *P. corymbosa* and *P. kotschyana* fruits include sesquiterpene hydrocarbons, while the essential oils of *P. aromatica* consist of phenylpropanoids.

According to the combined tree, *P. isaurica*, *P. flabellifolia*, *P. peucedanifolia* and *P. aurea* are a paraphy-

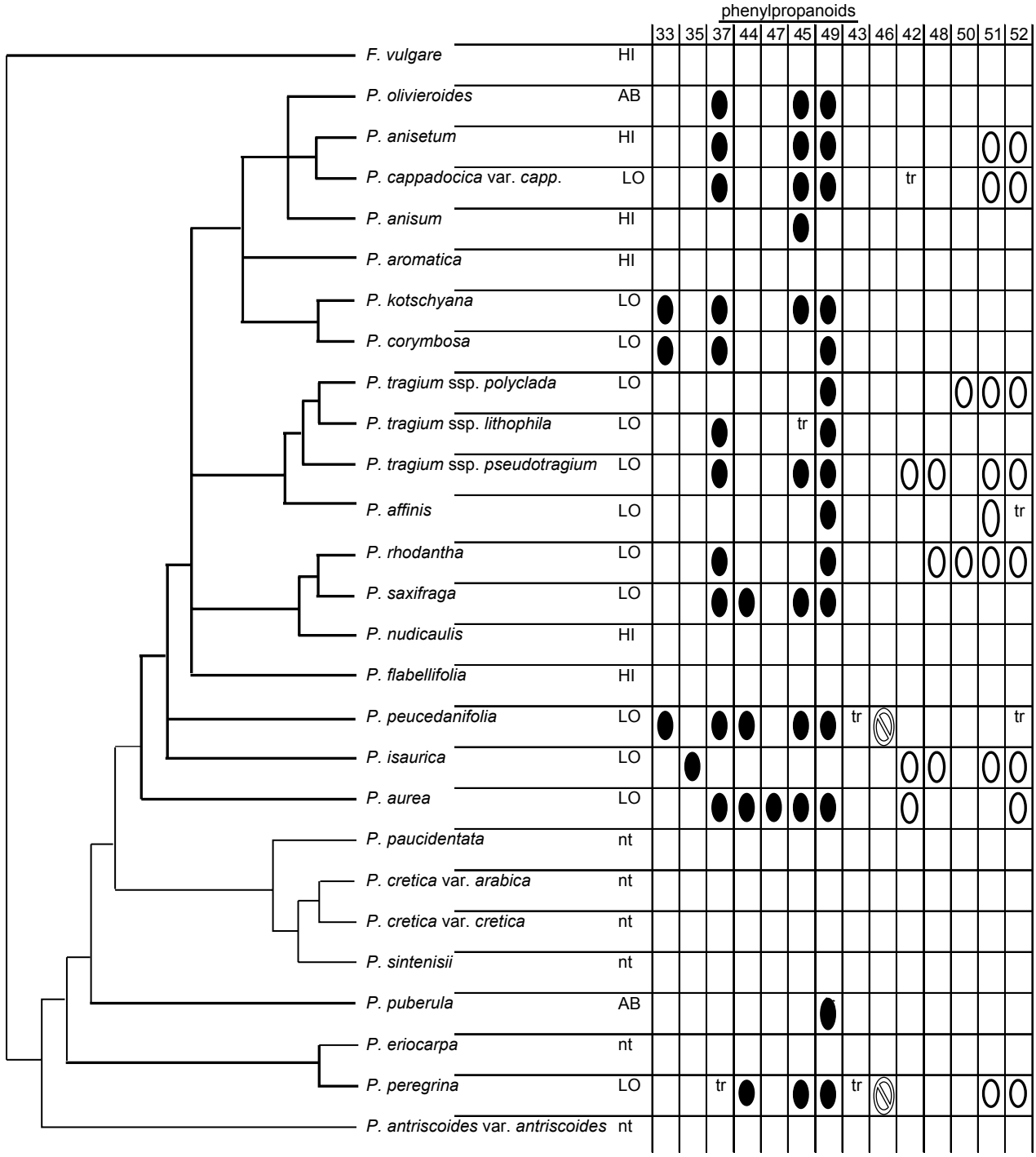

Fig. 4. Cladogram of *Pimpinella* illustrating the distribution of anethole and distributional relationship among PSIP compounds. Taxa in clades with thin branches denote taxa that were not chemically sampled (nt). Taxa with different concentrations of anethole are listed as having high (HI) concentrations or low (LO) concentrations. AB denotes taxa with no anethole isolated. The table to the right illustrates the presence of particular compounds (see Table 6) among taxa. The solid ovals represent chemicals positively associated with another, whereas empty ovals represent another group of chemicals associated with one another. The hatched ovals represent isolated compounds that show no positive association with any others. Trace (tr) $\leq 0.1\%$. The phenylpropanoid numbers represent the compounds in Tables 2–4.

Table 6. Statistics of phenylpropanoids

	33	35	37	42	43	44	45	46	47	48	49	50	51	52
33	xxx	0.952	0.004	0.910	0.203	0.120	0.031	0.611	0.121	0.823	0.111	0.886	0.771	0.722
35	0.941	xxx	0.741	0.045	0.876	0.923	0.796	0.712	0.932	0.112	0.381	0.076	0.120	0.162
37	0.040	0.651	xxx	0.210	0.021	0.081	0.001	0.891	0.421	0.320	0.052	0.500	0.363	0.3430
42	0.800	0.075	0.241	xxx	0.289	0.187	0.423	0.874	0.810	0.025	0.471	0.187	0.026	0.368
43	0.198	0.931	0.00031	0.118	xxx	0.0058	0.0038	0.827	0.187	0.693	0.012	0.810	0.662	0.608
44	0.135	0.947	0.011	0.091	0.008	xxx	0.036	0.782	0.135	0.782	0.131	0.864	0.761	0.692
45	0.019	0.875	0.00003	0.151	0.0024	0.019	xxx	0.862	0.271	0.564	0.018	0.712	0.340	0.428
46	0.712	0.713	0.715	0.812	0.792	0.856	0.849	xxx	0.0002	0.785	0.712	0.762	0.812	0.856
47	0.137	0.947	0.215	0.762	0.205	0.137	0.333	0.0003	xxx	0.787	0.113	0.862	0.743	0.710
48	0.819	0.075	0.621	0.002	0.745	0.819	0.527	0.628	0.819	xxx	0.482	0.0058	0.0005	0.0439
49	0.159	0.651	0.003	0.696	0.333	0.160	0.095	0.752	0.159	0.444	xxx	0.335	0.123	0.410
50	0.870	0.052	0.736	0.871	0.796	0.873	0.095	0.762	0.873	0.0073	0.440	xxx	0.010	0.016
51	0.471	0.950	0.270	0.048	0.289	0.473	0.652	0.785	0.473	0.021	0.341	0.085	xxx	0.004
52	0.775	0.105	0.270	0.112	0.658	0.775	0.422	0.754	0.775	0.035	0.44	0.012	0.00001	xxx

The numbers in bold represent phenylpropanoids found in *Pimpinella* species and reported in Tables 2–4.

letic group. They are morphologically quite different from one another and each species can be distinguished by basal leaves. The basal leaves of an endemic species *P. flabellifolia* are flabellate and the chemical composition is also different from *P. isaurica*, *P. peucedanifolia* and *P. aurea*. The composition of fruit, aerial parts (without fruits) and root essential oils of *P. flabellifolia* are much simpler and dominated by (*E*)-anethole and linalool (Tables 2–4). *P. peucedanifolia* can be easily distinguished from other *Pimpinella* species by its lanceolate to linear segments and with large toothed basal leaves. The main component of both the fruit and aerial parts (without fruits) is an *n*-alkane-type hydrocarbon: undecane (76.5 and 65.1%, respectively). It is not detected in the other *Pimpinella* oils. The root oil contains mainly epoxypseudoisoeugenyl 2-methyl butyrate (82.6%) (Table 4). The basal leaves of *P. isaurica*, an endemic, are coriaceous rather than leaf-like. The main constituents of the fruit oil of *P. isaurica* were α-zingiberene (15.7%) and a new phenylpropanoid, 4-(2-propenyl)-phenyl angelate (**35**) (13.7%) (Tabanca *et al.*, 2003). In the aerial parts (without fruits) and root essential oils of *P. isaurica*, the proportion of 4-(2-propenyl)-phenyl angelate and 4-(1-propenyl)-phenyl tiglate (**42**) showed a different proportion (43.3 and 12.6%, and 10.8 and 16.1%, respectively) in comparison to fruit oil (Tables 3 and 4). Compound **35** was not observed in the other essential oils of *Pimpinella*. The morphology of *P. aurea* is quite distinct with ovate-oblong basal leaves, deeply incised and large leaf lops and yellow flowers. The major compounds were (33.5%) a new epoxybisabolol-type oxygenated sesquiterpene named 1-methyl-4-(5-methyl-1-methylene-hex-4-enyl)-7-oxa-bicyclo [4.1.0] heptane (**30**) (33.5%) (Tabanca *et al.*, 2003) and β-bisabolene (33.1%). A new phenylpropanoid, 4-(3-methyloxiranyl)-2-methyl butyrate (**44**) (Tabanca *et al.*, 2005), was also isolated from the root oil (Table 4). Both the oil and DNA fingerprints of *P. aurea* are different from other species.

DNA sequence data show that *P. nudicaulis* is weakly linked with *P. rhodantha* and *P. saxifraga*. Although *P. nudicaulis* is morphologically similar to *P. saxifraga*, the compositions of their essential oils are very different. The essential oils obtained from fruit, aerial parts (without fruits) and roots of *P. nudicaulis* contained (*E*)-anethole (63.5, 27.8 and 12.5%, respectively) and *trans*-isoosmorhizole (**34**) (20.5, 12.2 and 78.9%, respectively) (Tables 2–4). Compound **34** is new for the genus *Pimpinella* (Tabanca *et al.*, 2004).

Matthew (1972) had trouble distinguishing transcaucasian *P. rhodantha* and *P. saxifraga* but the Turkish material of the two species can be distinguished based on the colour of their flowers (pink and white, respectively), and differences in the basal and cauline leaves. Therefore, the

specific status of *P. rhodantha* is maintained in the Flora of Turkey. The sequence data strongly support *P. rhodantha* and *P. saxifraga* as sister taxa but each has very different chemical profiles. (*Z*)-β-Farnesene (34.6%) and β-bisabolene (32.8%) were found as main compounds in the fruit oil of *P. rhodantha*, while sabinene (40.7%) and β-pinene (20.6%) were found as major constituents of *P. saxifraga*. Thus, these chemical compounds can be used to distinguish *P. rhodantha* and *P. saxifraga*. 4-Methoxy-2-(3-methyloxiranyl)-phenyl angelate (**51**) and 4-methoxy-2-(3-methyloxiranyl)-phenyl tiglate (**52**) were found as chief components in the root oil of *P. rhodantha* (each 29.1%) (Table 4). The root oil of *P. saxifraga* has a large amount of epoxypseudoisoeugenyl 2-methyl butyrate (66.6%), and minute amounts of other phenylpropanoids.

P. affinis and the *P. tragium* group subspecies (ssp. *lithophila*, ssp. *polyclada* and ssp. *pseudotragium*) are supported as a monophyletic group (Fig. 2). Morphologically, the *P. tragium* group subspecies are quite different from *P. affinis*. C_{12} hydrocarbons (geijerenes and azulenes, Fig. 1) derived from sesquiterpenes were found to be characteristic constituents of most *Pimpinella* oils. Two isomers, geijerene (**10**) and pregeijerene (**12**), are present as major components in the essential oil of both *P. affinis* and *P. tragium* ssp. *lithophila* (geijerene, 58.7 and 22.9%, respectively; pregeijerene, 19.5 and 5.0%, respectively). Both of these compounds have been hypothesized to be precursors of 1,4-dimethylazulene (**39**) (Kubeczka and Ullmann, 1980; Kubeczka *et al*., 1989; Kubeczka, 1997). Therefore compounds **10** and **12** can be used as chemotaxonomic markers to differentiate *P. affinis* and *P. tragium* ssp. *lithophila*. The azulene derivatives, responsible for the characteristic blue colour of the essential oils, are encountered rarely in nature. The phytochemical studies performed on *P. tragium* ssp. *lithophila* resulted in the isolation of two azulene derivatives, dictamnol (**32**) and a new azulene (**27**) named 4-(6-methyl-bicyclo[4.1.0]hept-2-en-7-yl)-butan-2-one (each $<5.0\%$) (Table 4). The major compounds were (*Z*)-β-farnesene (57.3 and 22.5%) in the fruit and aerial parts (without fruits) oil, 4-methoxy-2-(3-methyloxiranyl)-phenyl angelate (39.9%) and 4-methoxy-2-(3-methyloxiranyl)-phenyl tiglate (12.1%) in the root oil of *P. tragium* ssp. *polyclada*. β-Bisabolene (18.8%), γ-himachalene (12.4%) and α-pinene (15.7%) were dominant in the fruit oil of *P. tragium* ssp. *pseudotragium*. α-Pinene (30.5%) was a major component in the aerial parts (without fruits) oil, while root oil was rich in pseudoisoeugenol-type phenylpropanoids such as 4-methoxy-2-(3-methyloxiranyl)-phenyl angelate (30.7%) (**51**) and epoxypseudoisoeugenyl 2-methyl butyrate (18.8%) (**49**) (Table 4). It is noteworthy that *P. tragium* ssp. *lithophila* is chemically quite different from the other subspecies of *P. tragium*.

P. peregrina and *P. eriocarpa* are sister taxa in our analyses even though they are morphologically different. *P. peregrina* is biennial, while *P. eriocarpa* is an annual. Due to insufficient material, the essential oil of *P. eriocarpa* could not be obtained. The fruit oil and aerial parts (without fruits) of *P. peregrina* are rich in oxygenated sesquiterpenoid, *trans*-β-bergamotene (70.3 and 40.9%, respectively), while 4-methoxy-2-(3-methyloxiranyl)-isobutyrate (44.8%) (**46**) was the chief compound in the root oil of *P. peregrina* (Tables 3 and 4). The root oil of *P. puberula* could not be obtained due to insufficient material.

An annual species, *P. puberula*, fruit 1–1.5 mm long, ovoid-globose with dense spreading hairs, is phylogenetically isolated between the *P. eriocarpa*–*P. peregrina* clade and the other 22 taxa sampled. Limonene (63.4 and 36.5%) and methyl eugenol (23.1 and 29.6%) were major compounds in the fruit and aerial parts (without fruits) (Tables 2 and 3).

P. cretica Poiret (annual) was divided into two varieties based on leaf; var. *cretica* and var. *arabica* (Boiss.) Boiss. They form a monophyletic group in a four-taxon clade including perennial *P. sintenisii* and annual *P. paucidentata*. For *P. cretica*, *P. paucidentata*, *P. sintenisii* and *P. antriscoides* we were unable to obtain the essential oils due to insufficient material.

Biosynthesis of phenylpropanoids and norsesquiterpenes

Analysis of chemical profiles of the essential oils obtained from *Pimpinella* species used in this study shows a strong relationship between the pseudoisoeugenol and propenylphenol derivatives. This is not surprising since both classes of compounds appear to share the same biosynthetic pathway from L-phenylalanine to the formation of a *p*-propenylphenol (Fig. 5).

Retrobiosynthetic NMR analysis of pseudoisoeugenols in *Pimpinella* (Reichling and Martin, 1990, 1991; Reichling *et al*., 1995; Kubeczka, 1997) suggested that the pathway initiates with L-phenylalanine, which is first converted to *trans*-cinnamic acid then to *p*-coumaric acid via p-hydroxylation catalysed by the P450 cinnamic acid 4-hydroxylase. Concomitant reduction of the carboxyl group and methylation of the phenoxy group of *p*-coumaric acid by an *S*-adenosylmethionine-dependent *O*-methyltransferase yields the obligatory intermediate (*E*)-anethole (Reichling and Martin, 1990). Introduction of the second hydroxyl group (most likely by a P450 monooxygenase) is accompanied by a NIH-shift of the propenyl side chain, resulting in the 1-(*E*)-propenyl-2-hydroxy-5-methoxy benzene skeleton of pseudoisoeugenol (Martin and Reichling, 1992). While many derivatives of pseudoisoeugenol were observed in the *Pimpinella* species analysed (Fig. 5), this intermediate

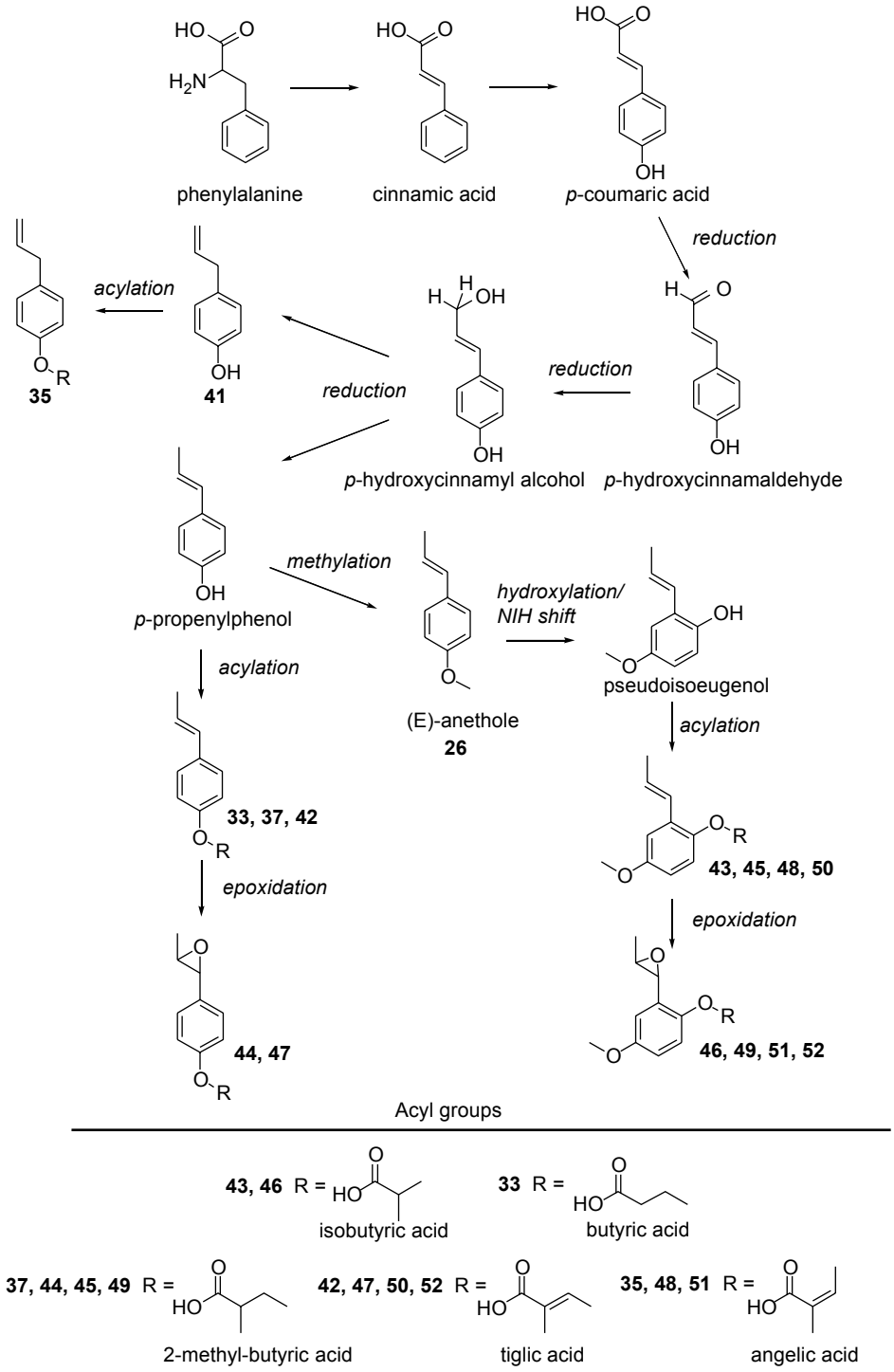

Fig. 5. Biosynthetic pathway of phenylpropanoids. The numbers in bold represent the compounds in Tables 2–4.

was not found. All of the pseudoisoeugenol analogues identified in the essential oils of the *Pimpinella* species included acylation with either isobutyric acid (**43**), 2-methyl-butyric acid (**45**), angelic acid (**48**) or tiglic acid (**50**), and their respective epoxidated analogues at the propenyl double bond of pseudoisoeugenol (**46**, **51** and **52**).

While acylation on pseudoisoeugenol of *Pimpinella* has been reported with other natural products, the enzymes responsible for this step (most likely acyl transferases) are not well characterized. Similarly, epoxidation of the propenyl double bond is not fully characterized, but it is expected to be catalysed by a P450 monooxygenase.

Biosynthesis of the disubstituted propenylphenol basically follows the same initial steps up to the formation of *p*-hydroxycinnamyl alcohol. Reduction of the alcohol forms a *p*-propenylphenol intermediate that is subsequently acylated with butyric acid (**33**), 2-methyl-butyric acid (**37**) or tiglic acid (**42**). Similarly to the pseudoisoeugenol derivatives, the propenyl side chain can be oxidized to form the epoxide congeners (**44** and **47**). It is interesting that no derivative with angelic acid side chains was identified in this group of compounds. Finally, a variation in the reduction of the cinnamyl alcohol can also result in an allyl side chain that leads to chavicol (**41**) and its angeloyl derivative (**35**).

The phyletic relationship among the phenylpropanoids points to differentiation of certain species. Typically, species with high levels of anethole (**26**) (i.e. *P. anisum*, *P. aromatica*, *P. nudicaulis* and *P. flabellifolia*) have little or no acylated anethole derivatives (Fig. 5). Conversely, species with a low level of (*E*)-anethole have more of the acylated derivatives (both propenylphenols and pseudoisoeugenols). From a biochemical and physiological stand point, our data indicate that all *Pimpinella* species have the ability to synthesize phenylpropanoids up to (*E*)-anethole. Species lacking the genetic and biochemical traits to modify this common precursor accumulate high levels of this metabolite. The other species making various acylated and epoxydated derivatives typically have low (*E*)-anethole concentration, suggesting that the production of (*E*)-anethole becomes rate limiting.

The biosynthesis of azulenes and geijerenes originates from elemane and there is some evidence that azulenes are derived from geijerenes (Kubeczka *et al.*, 1989). The phylogenetic relationship between the azulenes and geijerenes is not very clear. Nonetheless, species rich in azulenes (i.e. *P. tragium* ssp. *lithophila*, *P. affinis* and *P. corymbosa*) also have geijerenes (Fig. 3), underscoring their shared biosynthetic origin. However, there is little similarity between the species' relationships and their azulene and geijerene profiles.

Conclusion

This study provides useful information on the distribution of secondary metabolites in species of *Pimpinella* and, importantly, using a phylogenetic framework provides a working hypothesis of the relationships between certain essential oils and their putative biosynthetic pathways. (*E*)-Anethole was a major compound in the essential oils of *P. anisum*, *P. anisetum*, *P. flabellifolia* and *P. nudicaulis*, while other species accumulated metabolically modified derivatives, suggesting that the oil compositions can be used as an additional mode for classifying these species. The results indicate that oil composition of the *Pimpinella* species could be controlled by relatively few genes (enzymes) responsible for acylation and epoxidation of the phenylpropanoids. Importantly, this work also identified *P. anisetum*, *P. flabellifolia* and *P. nudicaulis* as potential alternative sources of (*E*)-anethole for the pharmaceutical industry.

This study provides a starting point to evaluate systematic relationships within *Pimpinella*. ITS and plastid-specific gene sequence comparison offers an important technique to define taxonomic relationships within the genus. Additional taxonomic sampling, particularly from other geographic regions, will improve our understanding of relationships as well as the phylogenetic significance of variability found within the genus. A phyletic framework is helpful in examining the putative relationships among secondary compounds and the evolution of diversity in biosynthesis of essential oils in these species. Additional studies should increase the taxonomic breadth as well as examine the life history and ecological roles responsible for the distributions of the chemicals. Precursory examinations of the life history and ecology of the different species of *Pimpinella* did not reveal any underlying external influences on patterns of the chemical distributions. This present study provides genetic and chemical markers for authentic identification of *Pimpinella* species. From a chemotaxonomic point of view, the presence of norsesquiterpenes and phenylpropanoids in *Pimpinella* separates it from all the other *Apiaceae* investigated thus far.

Acknowledgements

We acknowledge Prof. Dr B. Yildiz, Assoc. Dr A. Sokmen, Assist. Dr E. Sonmez, Assist. Dr M. Ekici, Dr H. Akan, Res. Assist. F. Karaveliogullari, Dr S. Celik, Dr T. Tugay, Dr S. Terzioglu, Res. Assist. T. Arabaci, Res. Assist. T. Degirmenci, Mr I. Yaman, Mr I. Koklu, Mr I. Yilmaz and Mr A. Koruk for help with collection of plant materials. We thank Dr B. Demirci and Dr T. Ozek for GC-MS analysis of the essential oils. We would like to express our thanks to Prof. Dr I. Hedge, Royal Botanic Garden, Edinburgh, for supplying *P. cretica* var. *cretica*, *P. cretica* var *arabica*, *P. sintenisii* and *D. antriscoides* var. *antriscoides*. We acknowledge M. Ruscoe, A. Prause and A. Michel for their laboratory expertise. This research was supported by Anadolu University Research Fund, Eskisehir, Turkey (AUAF 00 03 34) and in part by the

United States Department of Agriculture, Agricultural Research Service Specific Cooperative Agreement No. 58-6408-7-012.

References

Alman AH, Jawad ALM, Al-Bana AS and Ali KF (1988) Antiviral activity of some Iraqi indigenous plants. *International Journal of Crude Drug Research* 26: 107–111.

Baser KHC (1997) *Tibbi ve aromatik bitkilerin ilac ve alkollu icki sanayinde kullanimi*. Istanbul: Istanbul Ticaret Odasi yayinlari, pp. 33–35.

Baser KHC, Ozek T, Duman H and Guner A (1996) Essential oil of *Pimpinella aromatica* Bieb. from Turkey. *Journal of Essential Oil Research* 8: 463–464.

Bisset NG (1994) *Herbal Drugs and Phytopharmaceuticals*. London: CRS Press, pp. 72–75, 375–377.

Bohn I, Kubeczka KH and Schultze W (1989) The essential root oil of *Pimpinella major*. *Planta Medica* 55: 489–490.

Branther A and Grein E (1994) Antibacterial activity of plant extracts used externally in traditional medicine. *Journal of Ethnopharmacology* 44: 35–40.

Constance L (1971) History of the classification on Umbelliferae (Apiaceae). In: Heywood VH (ed.) *The Biology and Chemistry of the Umbelliferae*. New York: Academic Press, pp. 109–155.

Crellin JK and Philpott J (1990) *Herbal Medicine Past and Present*. Durham, NC: Duke University Press, pp. 58–59.

Downie SR, Ramanath S, Katz-Downie DS and Llanas E (1998) Molecular systematics of Apiaceae subfamily Apioideae: phylogenetic analysis of nuclear ribosomal DNA internal transcribed spacer and plastid rpoC1 intron sequences. *American Journal of Botany* 85: 563–591.

Downie SR, Katz-Downie DS and Spalik K (2000a) A phylogeny of Apiaceae tribe Scandiceae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *American Journal of Botany* 87: 76–95.

Downie SR, Katz-Downie DS and Watson MF (2000b) A phylogeny of the flowering Apiaceae based on chloroplast DNA rpl16 and rpoC1 intron sequences: towards a suprageneric classification of subfamily Apioideae. *American Journal of Botany* 87: 273–292.

Downie SR, Watson MF, Spalik K and Katz-Downie DS (2000c) Molecular systematic of old world Apioideae (Apiaceae): relationship among some members of tribe Peucedaneae Sensu Lato, the placement of several island-endemic species and resolution within the Apioid Superclade. *Canadian Journal of Botany* 78: 506–528.

Downie SR, Plunkett GM, Watson MF, Spalik K, Katz-Downie DS, Valiejo-Roman CM, Terentieva EI, Troitsky AV, Lee BY, Lahham J and El-Oqlah A (2001) Tribes and clades within Apiceae subfamily Apioidea: the contribution of molecular data. *Edinburgh Journal of Botany* 58: 301–330.

Drude O (1897-98) Umbelliferae. In: Engler A and Prantl K (eds) *Die naturlichen pflanzenfamilien*, Vol. 3. Leipzig: Wilhelm Engelmann, pp. 63–250.

Duke JA (1985) *Handbook of Medicinal Herbs*. London: CRC Press, pp. 374–375.

Grieve M (1970a) *The Medicinal, Culinary, Cosmetic and Economic Properties, Cultivation and Folk-lore of Herbs, Grasses, Fungi, Shrubs and Trees*, Vol. 1. New York: Hafner Publishing Co., pp. 41–43.

Grieve M (1970b) *The Medicinal, Culinary, Cosmetic and Economic Properties, Cultivation and Folk-lore of Herbs, Grasses, Fungi, Shrubs and Trees*, Vol. 2. New York: Hafner Publishing Co., pp. 146–147, 720–721.

Haranath PS, Akther MH and Sharif SI (1985) Acetylcholine and choline in common spices. *Phytotherapy Research* 1: 91–92.

Hasegawa M, Kishino H and Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22: 160–174.

Kiuchi F (1995) Studies on the nematodical constituents of natural medicines. *Natural Medicines* 49: 364–372.

Kollmannsberger H, Fricke G, Paulus H and Nitz S (2000) The flavor composition of umbelliferous fruits. Part 1. Anise (*Pimpinella anisum*). *Advances in Food Sciences* 22(1/2): 47–61.

Kleiman R and Spencer GF (1982) Search for new industrial oils. *Journal of the American Oil Chemists' Society* 59: 29–32.

Kleiman R, Plattner DR and Weisleder D (1988) Antigermination of phenylpropanoids from the genus *Pimpinella*. *Journal of Natural Products* 52: 249–256.

Kubeczka KH (1997) The essential oil composition of *Pimpinella* species. In: Baser KHC and Kirimer N (eds) *The 28th International Symposium on Essential Oils, Proceedings, 1–3 September, Eskisehir, Turkey*. Eskisehi Anadolu University, pp. 35–56.

Kubeczka KH and Ullmann I (1980) Occurrence of 1,5-dimethylcyclodeca-1,5,7-triene (pregeijerene) in *Pimpinella* species and chemosystematic implications. *Biochemical Systematics and Ecology* 8: 39–41.

Kubeczka KH, Bohn I and Wuerzburg F (1985) Thin layer and gas chromatography, structure revision of the principal components of the essential oils. *Deutsche Apoteker Zeitung*, 125: 399–402.

Kubeczka KH, Bohn I and Schultze W (1989) The compositions of the essential roots oils from *Pimpinella saxifraga* L. and chemotaxonomic implications. *Zeitschrift für Naturforschung* 44: 177–182.

Lokar LC and Poldini L (1988) Herbal remedies in the traditional medicine of the Venezia Giulia. *Journal of Ethnopharmacology* 22: 231–278.

Maddison DR and Maddison WP (2003) *MacClade 4.06—Analysis of Phylogeny and Character Evolution*. Sunderland, MA: Sinauer Press.

Marcus C and Lichtenstein EP (1979) Biologically active components of anise: toxicity and interactions with insecticides in insects. *Journal of Agricultural and Food Chemistry* 27: 1217–1223.

Martin R and Reichling J (1992) NIH-shift during biosynthesis of epoxypseudoisoeugenol-2-methylbutyrate in tissue cultures of *Pimpinella anisum*. *Zeitschrift für Naturforschung* 43: 42–46.

Matthew VA (1972) Pimpinella. In: Davis PH (ed.) *Flora of Turkey and East Aegean Islands*, Vol. 4. Edinburgh: Edinburgh University Press, pp. 352–364.

Miller LG and Murray WJ (1998) *Herbal Medicines*. New York: The Haworth Press, p. 74.

Pimenov MG and Leonov MV (2004) The Asian Umbelliferae Biodiversity Database (ASIUM) with particular reference to SW Asian taxa. *Turkish Journal of Botany* 28: 139–145.

Reichling J and Martin R (1990) Further studies on the biosynthesis of pseudoisoeugenols in tissue cultures of *Pimpinella anisum*. *Zeitschrift für Naturforschung* 45: 942–948.

Reichling J and Martin R (1991) Pseudoisoeugenol-eine gruppe seltener phenylpropanoide im genus *Pimpinella*: Biosynthese und biologische wirkung. *Pharmazeutische Zeitung-Wissenschaft* 45c: 225–231.

Reichling J and Merkel B (1991) Studies on the biological activities of rare phenylpropanoids of the genus *Pimpinella*. *Journal of Natural Products* 54: 1416–1418.

Reichling J, Martin R and Kemmeter B (1995) Biosynthesis of pseudoisoeugenol-derivatives in liquid tissue cultures of *Pimpinella anisum*. *Plant Cell, Tissue & Organ Culture* 43: 131–136.

Ross SA, El-Keltawi NE and Megalla SE (1980) Antimicrobial activity of some Egyptian aromatic plants. *Fitoterapia* 51: 201–205.

Shashikanth KN and Hosono A (1986) In vitro mutagenity of tropical species to *Streptomycin* dependent strains of *Salmonella typhimurium* TA 98. *Agricultural and Biological Chemistry* 50: 2947–2948.

Swofford DL (2001) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10*. Sunderland, MA: Sinauer Associates.

Tabanca N, Bedir E, Kirimer N, Baser KHC, Khan SI, Jacob MR and Khan IA (2003) Antimicrobial compounds from *Pimpinella* species growing in Turkey. *Planta Medica* 69: 933–938.

Tabanca N, Khan SI, Bedir E, Annavarapu S, Willett K, Khan IA, Kirimer N and Baser KHC (2004) Estrogenic activity of isolated compounds and essential oils of *Pimpinella* species from Turkey, evaluated using a recombinant yeast screen. *Planta Medica* 70: 728–735.

Tabanca N, Bedir E, Ferraaira D, Slade D, Wedge DE, Jacob MR, Khan SI, Kirimer N, Baser KHC and Khan IA (2005) Bioactive constituents from Turkish *Pimpinella* species. *Chemistry & Biodiversity* 2: 221–232.

Twaij HA, Elisha EE, Khalid RM and Paul NJ (1987) Analgesic studies on some Iraqi medicinal plants. *International Journal of Crude Drug Research* 25: 251–254.

Xue KF, Wang JZ and Wen YS (1992) A new flavone glucoside from *Pimpinella thellungiana*. *Chung Ts'Ao Yao* 29: 452–455.